IDENTIFICATION AND PROCUREMENT OF HIGH YIELDING YEAST CULTURE (STRAINS) FOR ETHANOL PRODUCTION FROM USE LESS BROKEN RICE

[1]O.P. Suryawanshi, [2]D. Khokhar

[1]SRF, NET, Ph.D., [2]Assistant Professor (IGAU, Raipur)
Department of Food Process Engineering,
National Institute of Technology Rourkela, Odisha
India – 769008.

***Abstract*: Fermentation is one of the crucial process to produce the ethanol from the bio-materials. The yield of fermented product may depend on the type of feed, starter culture, and fermenting parameters like time, temperature, agitation speed and nutrient supplements. Fermenting cultures may directly affect the type and yield of the of fermented product. The fermentation of agricultural and biomaterials under suitable condition may produce the ethanol and can helps in waste management. The production of ethanol greatly depends on the fermenting culture which may be either yeast, enzyme or fungal. The broken rice is one of the less preferably agriculture produce which poses least price in the market and generally uses for animal feed. The least price of broken rice gives affects the farmer's annual income. In this contrast the fermentation of broken rice may enhance the annual income of the farmers. But the fermentation of broken rice may produce both ethanol as well as methanol at the same time which may be bless or curse for human being. In this regarding the fermenting culture plays an important role during the ethanol production. To produce the ethanol from the broken rice its necessary to identifies the fermenting culture and their trains. In this research the fermenting yeast *Saccharomyces cerevisiae* and three strains namely NCIM 3570, NCIM 3281 and NCIM 3640 were identified and procured.**

***Keywords*: Broken rice, reducing, ethanol, *Saccharomyces cerevisiae*, enzymatic pre-treatment, anaerobic fermentation.**

INTRODUCTION

The present status of fossil fuels is degrading day by day. The complete exhaustion of the fossil fuels can stop the whole world. Natural resources like wood, water, wind, sun and coal are the main sources of energy in the world but least of them are renewable and rest are not. Present days almost all energies are coming from the fossil fuels and that is exhaustible which is burning problem of the world. The researchers are cautiously trying to find out the alternative sources of the fossil fuels. After a long time, research ethanol has been found as the alternative source which can produce mainly by fermentation. Fermentation of bio-materials is one of the key process to produces the ethanol. Fermentation may be a metabolic process or conversion of sugar into either ethanol or methanol During fermentation sugars are consumed either in the absence or presence of oxygen by various microorganisms that may be bacteria, fungal and yeast.In general fermentation is metabolic process of starch (simple sugar) which may produce gas, ethanol, methanol and other acids. The main sources of starch are generally biomaterials which is also an exhaustible sources of energy. Rice is one of the easily growing crops which contain starch as a major constituent in it. India is the second largest rice producer country after China in the world. Chhattisgarh is one the largest rice producer state among the Indian states and also known as the bowl of rice. Chhattisgarh is producing rice crop mainly in two seasons namely *rabi* seasons and *kharif* season, that increase the rice production day by dayin huge amount. Rice occupies average of 3.6 million hectares with the productivity of the state ranging between 1.2 to 1.6 t/ha. The rainfall, which is major factor determining rice productivity, is quite high with an irrigated area of nearly 28%. In Chhattisgarh rice is grown in irrigated areas during summer season with an area of 225-231 hectare with 548.28-577.05 MT productions (Annonymous, 2013). It has been found that there is increase in the rice production from Chhattisgarh in summer season.

Day time temperatures during peak summer season are usually very high in the entire area and peaking in the second fortnight of May. Summer season rice is grown in large area of Chhattisgarh and it is known that this type of rice has higher broken percentage on milling process in comparison to *kharif* grown rice. High temperature at the time of grain maturation leads to sudden drying that increases cracks in the grain. If harvested paddy is not processed properly in time spoilage occurs mainly due to microorganisms, causing deterioration in quality of rice. Losses are also incurred during storage due to rodents, insects, fungi and improper storage structures. The microbial infection converts part or whole of the gelatinized translucent kernel to opaque chalky rice, which broke upon milling. Sometimes the rice becomes unfit for human consumption or it is used for animal feeding. The broken rice obtained during milling has a value of (half to one-third) less than whole rice.

The raw starch material from the varieties, which are less preferred or not preferred commercially for regular use as staple food, can be used for the ethanol production. They are less preferred since the grains are medium bold to bold type i.e., not of fine grained or fine quality, the rice will be sticky after cooking or has high broken percentage.Considering all the above points, aim of present investigation was Identification and procurement of high yielding yeast culture (strains) for ethanol production from use less broken rice.

MATERIAL AND METHODS

Experiments were conducted pertaining to research investigation during 2014-2015 at the Department of Agricultural Processing and Food Engineering in collaboration with the Department Plant Physiology, Agriculture Biochemistry Medicinal and Aromatic Plant, Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.).Summer grown broken raw rice of different varieties were collected from the various rice mill units which are usually not fit for human consumption and poses negligible price in the market. After collection of broken rice the dirt particles, stems, stones and other filth were removed thoroughly. Cleaned broken rice was pre-treated to convert the rice starch into free sugars and pre-treated starchwere inoculated with the three different yeast cultures for ethanol production. The cultures were identified for maximum production of ethanol. The details of material and methods followed in this investigation are as follows:

Selection and procurement of rice varieties

The commonly summer grown rice varieties (viz. IR-36, IR-64, MTU-1010, Danteshwari, Mahamaya HMT, and Javafull etc.) of the Chhattisgarh state collected from the Department of Genetics and Plant Breeding, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur. The broken rice percentage was determined by availing the lab scale milling facilities available in Department of Genetics and Plant Breeding. After determination of broken percentage of rice varieties, the four rice varieties namely as: IR-36, IR-64, MTU-1010 and Danteshwari were selected for the study.

Selection and Procurement of Culture

Initially the cultures were listed from the list available on the websites of national repository (NCIM, Pune and MTCC, IM Tech. Chandigarh). Further ethanol producing capability was determined on the basis of available literature. Three yeast strain of *Saccharomyces cerevisiae viz.*NCIM 3570, NCIM 3281 and NCIM 3640 were selected and procured from the National Collection of Industrial Micro-organisms (NCIM), Pune, Maharashtra.
The microbial cultures used in the study are as following:

Table:1 Cultures procured for fermentation

S.N.	Name of the culture	Source
1.	*Saccharomyces cerevisiae* NCIM 3281	NCIM, Pune
2.	*Saccharomyces cerevisiae* NCIM 3570	NCIM, Pune
3.	*Saccharomyces cerevisiae* NCIM 3640	NCIM, Pune

Media Preparation

Yeast strains were maintained on MGYP media having a composition: malt extract – 0.3 g, glucose – 1.0 g, yeast extract – 0.3 g, Peptone – 0.5 g and agar-agar 2 g per 100 ml and YEPDA composition: yeast extract-1 g, peptone-2 g, dextrose-2 g, agar-agar-2 g per 100 ml. The pH maintained at 7.0 and the media was autoclaved at 121° C and 15 psi for 20 minute for the sterilization purpose.

Maintenance of cultures

Procured yeast cultures were revived separately (Plate1) on specified media in petri plates at 28±1°C in Remi make incubator. Further, single colony of the culture from the petri plate was inoculated in the 50 mL MGYP media or broth in 100 ml conical flask separately and incubated for 24h at 28±1°C. The 24h grown cultures were used as mother culture/pre-culture. Mother cultures were again inoculated in the broth to find out the log phase (optimum growth stage) of the culture. Graph was plotted time vs optical density (OD). The OD was read at 640 nm using a Systronics make single beam spectrophotometer. After determination of the log phase (Fig. 1 to 3) of all the culture the cultures were used as inoculums in all the experiment only from the specified time (log phase).

Plate:1 Revival of *S. cerevisiae* of different strain on media

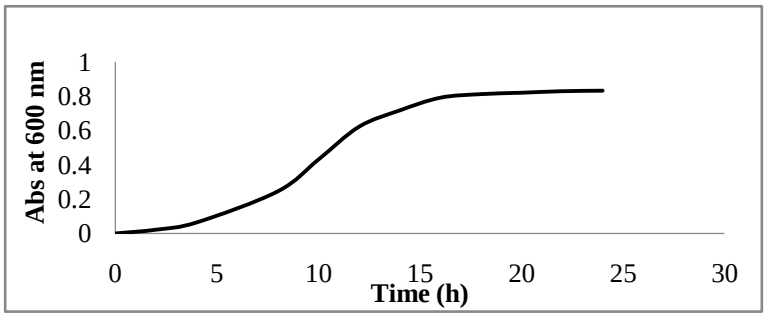


Fig. 1 Growth curve for *Saccharomyces cerevisiae* NCIM 3281

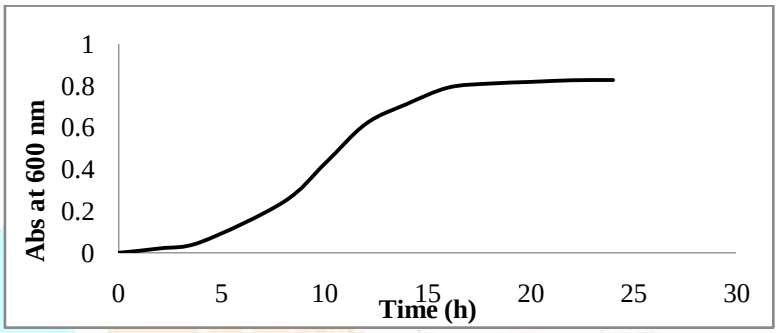


Fig. 2 Growth curve for *Saccharomyces cerevisiae* NCIM 3570

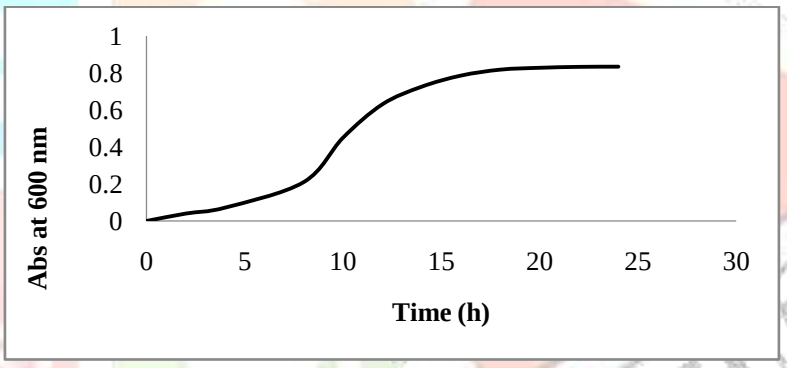


Fig. 3 Growth curve for *Saccharomyces cerevisiae* NCIM 3640

Preparation of the substrate

A known quantity (50 gm) of each rice variety (IR-36, IR-64, MTU-1010 and Danteshwari) was steep for one hour and cooked separately in aluminium cooker having 1Ltr. capacity with equal amount of water (W/V) up to 5 min after one whistle on sim mode. After cooling of the cooked rice, paste was prepared using pastel mortar. Further, 25 gm of the mashed (paste) substrate weighed separately and volume was made to 35 ml with distilled water for the hydrolysis of fermentable sugars.

Preparation for pre-treatments

Acid pre-treatment

The mashed substrate was pre-treated with 25 ml sulphuric acid (Plate 3.2) at different concentrations *viz.*, 0.5, 1.0, 2.0 and 2.5 per cent and kept at different incubation periods *viz.*, 2, 4, 8, and 24 hours at 28±2°C for hydrolysis of fermentable sugars.

Enzyme pre-treatment

Commercial α-amylase (Diastase α-amylase) enzyme was prepared with buffer, 10 mM $CaCl_2$ at different concentration viz., 0.5, 1.0 and 2.0 per cent and added to the mashed substrate for saccharification.

Period optimization for hydrolysis

The incubation period for hydrolysis of substrate was standardized for the time intervals of 1, 2, 3, 4, 5, 6 and 7 hours for commercial α-amylase and 2, 4, 8 and 24 hours for acid pre-treatment.

Estimation of sugars

Reducing sugar

The reducing sugars were estimated (Plate 3.3) by following 3, 5, Dinitrosalicylic acid method (Miller, 1959).

Preparation of reagents

i. DNSA: One gram of 3,5, Dinitrosalicylic acid (DNSA), 200 mg of crystal phenol and 50 mg of sodium sulphite was dissolved in 1.0% NaOH solution and the volume was made up to 100 mL reagent was stored at 4°C. Since the reagent deteriorates during long storage due to sodium sulphite; hence, sodium sulphite was added at the time of use.

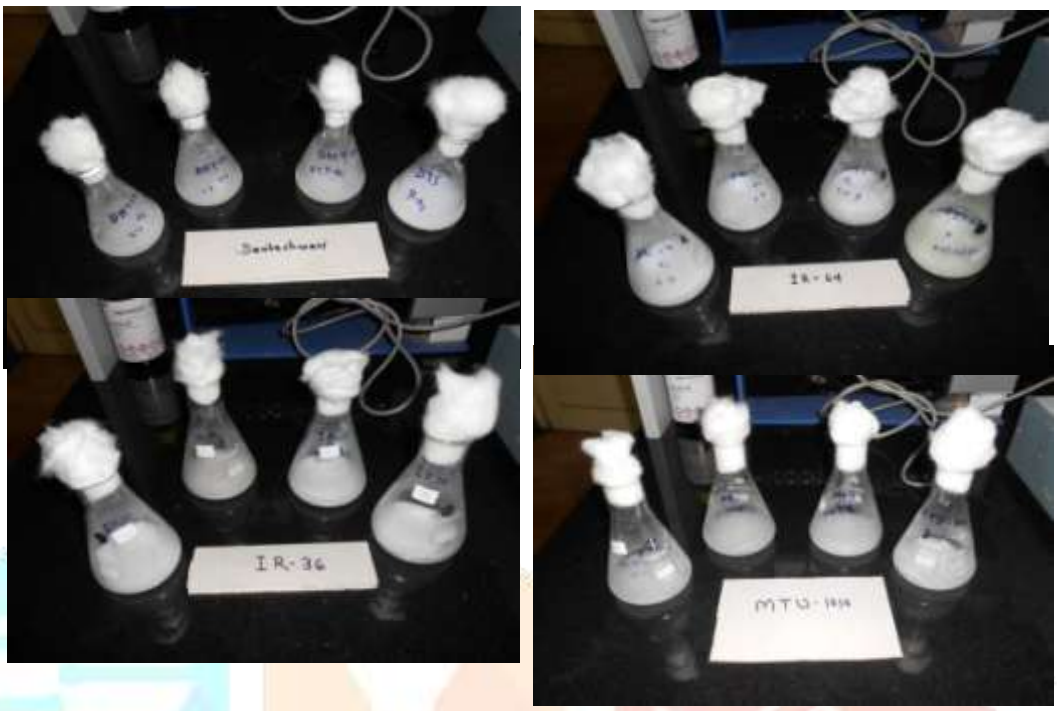


Plate:2Pre-treatment of broken rice of four varieties for fermentation process

Plate:3 Reducing sugar analysis by DNS method

ii. Rochelle salt solution 40%

Rochelle salt solution was prepared by dissolving 40 g of potassium sodium tartarate in distilled water and volume was made up to 100 ml.

iii. Preparation of stock solution of glucose

Standard stock solution of glucose was prepared at 1 mg/ml by dissolving 100 mg of D-glucose in distilled water and final volume was made upto 100 ml.

Procedure

Sample of 0.5 ml from acid pre-treated and 0.1 ml from enzymatic pre-treated hydrolyzed sample was drawn from each treatment and delivered into thin walled test tubes and volume was made to 1.0 ml with distilled water. The reagent blank containing 1 ml of distilled water was also kept. Similarly, standards were also included ranging from 0.1 mg to 1.0 mg/ml of glucose. 0.5 ml of DNSA reagent was added to each sample, mixed well and kept on boiling water bath for 5 min. The sample was added with 1 ml of 40 per cent Rochelle salt solution before cooling and volume was made upto 25 ml using volumetric flask.Absorbance in terms of optical density of the standard and the sample were recorded at 510 nm using visible spectrophotometer-106 (Plate 4). The standard curve of glucose was plotted on graph (Fig. 4).

Plate: 4 Spectrophotometer-106

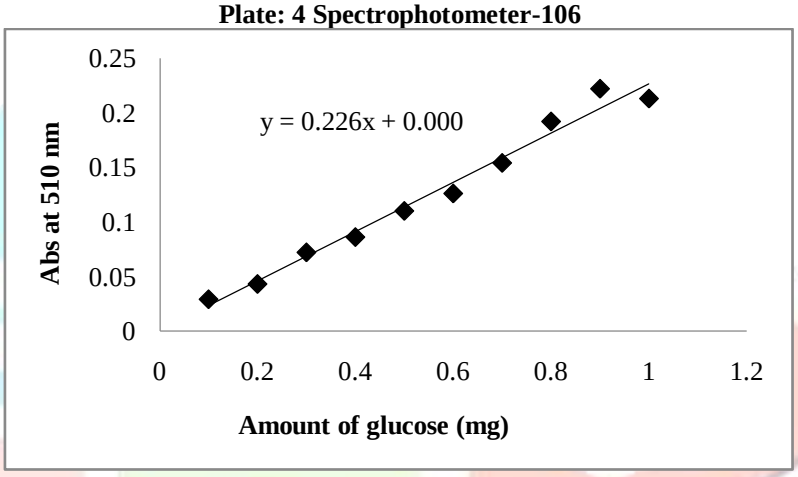


Fig. 4 Standard graph for glucose using DNSA method

Nutrient supplementation

The hydrolysate was supplemented with nitrogen and phosphorus in the form of diammonium hydrogen phosphate (0.5 g/l).

Protein estimation

Protein in the hydrolysate was estimated by modified Microkjeldhal method standardized by Jackson (1973). 0.5 gm of sample was digested with 10 ml of concentrated sulphuric acid along with 0.2 g catalyst mixture K_2SO_4:$CuSO_4$: Selenium (100:10:1) after cooling the distillation was carried out in semi-Microkjeldhal unit. The ammonia evolved was trapped in 4 per cent boric acid containing mixed indicator (Bromo cresol green, 0.066 g and methyl red, 0.033 g in 100 ml methanol). It was titrated against 0.5 N sulphuric acid and total N content of the hydrolysate was determined and the results were expressed as per cent of N. Total protein was estimated by multiplying conversion factor 5.95 into nitrogen and expressed in per cent.

Estimation of starch

The starch was estimated by anthrone method (Hodge and Hofreiter, 1962).

Preparation of Reagents

i. Anthrone reagent

Two hundred mg of anthrone powder was dissolved in 100 ml of ice cold 95 per cent sulphuric acid.

ii. Preparation of stock solution of glucose

Standard stock solution was prepared by dissolving 10 mg of D-glucose in distilled water and final volume was made upto 10 ml with distilled water.

Procedure

Homogenize well-grounded rice sample of 0.5 g in hot 80% ethanol to remove sugars. Centrifuge and retain the residue repeatedly with hot 80% ethanol till the washing does not give color with anthrone reagent. To the residue add 0.5 ml of water and 6.5 ml of 52% perchloric acid. Extract at 60°C for 20 min. Centrifuge and collect the supernatant. Repeat the extraction using fresh perchloric acid. Centrifuge and collect the all the supernatant and makeup upto 100 ml. Pipette out the 0.2 ml of the

supernatant and make up the volume to 1 ml with water. Prepare the glucose standard by taking 0.2, 0.4, 0.6, 0.8 and 1ml of standard solution of glucose. Add 4 ml of anthrone reagent to each tube. Heat the sample for eight minutes in boiling water bath. The samples were cooled rapidly and the colour intensity of the standards and the samples were recorded as 630 nm using visible spectrophotometer-106. The standard curve of glucose was plotted on graph (Fig. 5).

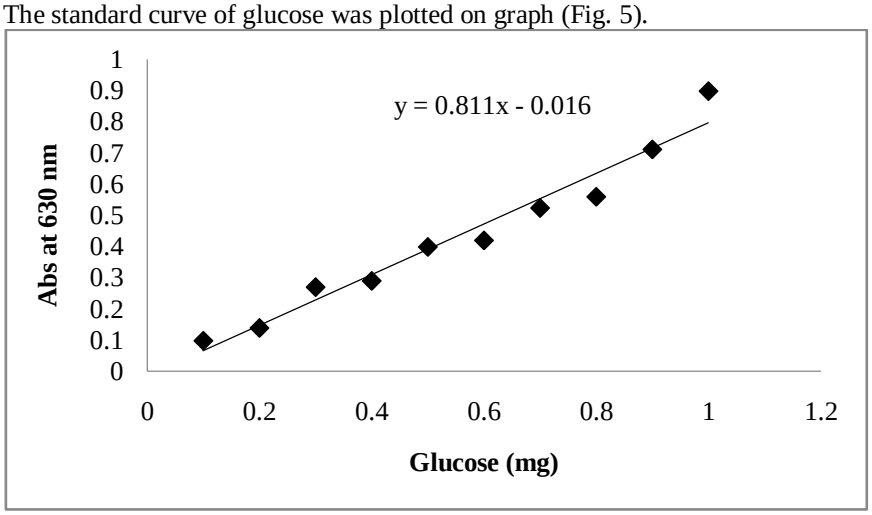


Fig. 5 Standard graph of glucose using Anthrone reagent

Fermentation

After hydrolysis of samples volume was made up upto 100 ml for fermentation. The hydrolysate from the pre-treatment was ameliorated to obtain 24°Brix by adding cane sugar. Brix reading of the samples was determined with the help of hand refractometer having a range of 0-32°Brix at 20°C and pH was adjusted to 3.5 by adding sodium bicarbonate. Activity of the natural flora of the must was suppressed by adding 200 mg of potassium metabisulphite and kept for 4-5 hours. The must was supplemented with diammonium hydrogen phosphate (0.5 g/l) as a source of nitrogen and phosphorus.

The pre-treated samples (100 ml) of rice varieties were inoculated with standard yeast, *Saccharomyces cerevisiae* 3281, *Saccharomyces cerevisiae* 3570 and *Saccharomyces cerevisiae* 3640 @ 5 per cent. The samples were fermented anaerobically at 28±1°C in incubator at 90 rpm.

Estimation of ethanol

The ethanol was estimated by colorimetric method as described by Caputi *et al*. (1968).

Preparation of reagent

i. Potassium dichromate solution

Thirty-four grams of $K_2Cr_2O_7$ was dissolved in 500 ml distilled water, 325 ml of sulphuric acid was added to it slowly and volume was made up to 1000 ml with distilled water to give 0.23N $K_2Cr_2O_7$.

ii. Preparation of standard ethanol solution

Standard ethanol solution was prepared by dissolving 12.67 ml of 100 per cent pure analytical grade (containing 789 mg/ml) ethanol in 100 ml distilled water, which results in 10 mg/ml of standard ethanol.

Procedure

One ml of representative samples from each treatment was transferred to 250 ml round bottom distillation flask connected to the condenser and was diluted with 30 ml distilled water. The sample was distilled at 74-75°C. The distillate was collected in 25 ml of 0.23 N $K_2Cr_2O_7$ reagents, which was kept at receiving end. The distillate containing ethanol was collected till total volume of 45 ml was obtained. Similarly, standards (20-100 mg ethanol) were mixed with 25 ml of $K_2Cr_2O_7$ separately and the volume was made up to 45 ml. The distillate of samples and standards were heated in water bath at 60°C for 20 minutes and cooled. The volume was made upto 50 ml with distilled water and the optical density was measured at 600 nm using visible spectrophotometer-106. The standard curve was plotted considering the concentration against absorbance.

RESULTS AND DISCUSSION

Ethanol is a fermented product of cereals, fresh fruits *etc*. ethanol from rice is produced after saccharification of starch by acids, enzymes (especially, commercial amylase) *etc*. Produced raw ethanol is a complex mixture of organic and inorganic substances like carbohydrates, proteins, amino acids, ethyl ethanol, organic acids, inorganic acids and micronutrients *etc*. The quality/ quantity of ethanol depend on the different yeast strains and also differs with rice varieties. The experimental results on screening of rice varieties and microbial cultures, standardization of pre-treatment methods for efficient hydrolysis for release of free sugar, screening of yeast strains for ethanol production and culture identification are presented as follows:

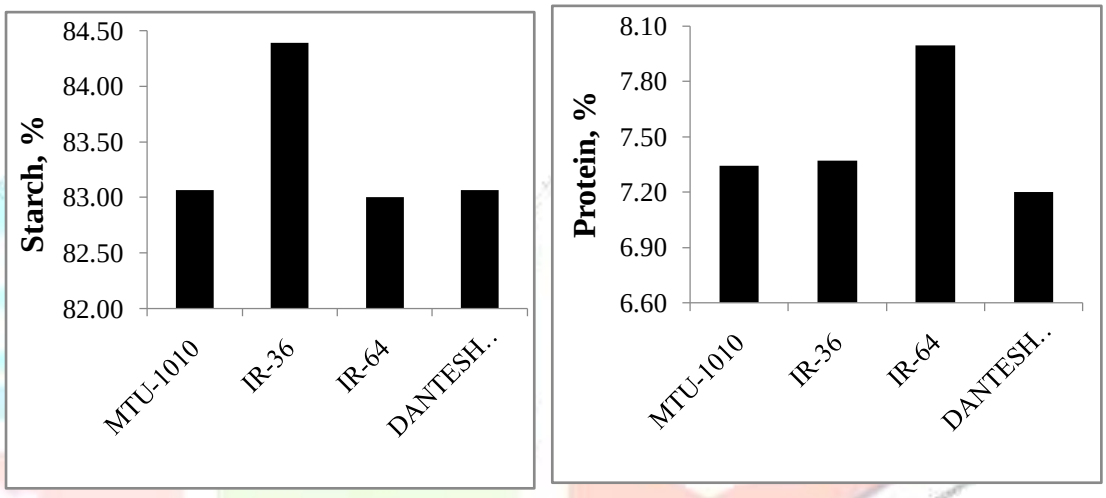
Initial starch and protein content of different rice varieties

The data recorded on starch and protein content in different selected varieties of rice are presented in Table 2 and Fig. 6(a & b) the obtained results clearly indicated that rice varieties differed in starch and protein contents. The highest starch content was recorded in IR-36 rice variety which accounts to 84.393 per cent, followed by MTU-1010 (83.067%) variety, which did not differ significantly with DANTESHWARI (83.067%) and IR-64 (83.003%) varieties. Highest protein content was recorded in IR-64 rice variety (7.997%) followed by IR-36 variety (7.370%) and both were significantly superior over other two rice varieties.

Ramarathnam and Kulkarni (1988) and Sadhana Singh *et al.* (1998) also observed wide variation in starch content (65-72%, 61.76%-77.95%) of 17 and 6 varieties, respectively. Damir (1985) reported that the parboiled and raw rice when milled contained crude protein of 8.14 and 7.67, respectively.

Table: 2 Initial starch and protein content in different rice varieties

S.N.	Rice varieties	Starch %	Protein %
1	MTU-1010	83.067	7.342
2	IR-64	83.003	7.997
3	IR-36	84.393	7.370
4	DANTESHWARI	83.067	7.200
		C.D. 0.581	C.D. 0.090
		SE(m)±0.175	SE(m)±0.027



(a) (b)

Fig. 6 Starch and Protein percentage of selected varieties

Effect of enzyme pre-treatment

Effect of commercial α-amylase (Diastase α-amylase) on hydrolysis

An experiment was conducted to know the effect of commercial α-amylase pre-treatment on hydrolysis on different rice varieties. Reducing sugar content of rice differed at different incubation periods along with different concentration of α- amylase enzyme *viz.* 0%, 0.5%, 1%, and 2% level.

Effect of Incubation period on releasing of reducing sugar in different rice varieties

It can be inferred from the Table 3 and Fig. 7 that maximum reducing sugar was released in IR-36 ranging from 12.078 to 47.278 mg/g at different incubation period, with the mean 34.135 which is significantly higher in comparison to other rice varieties. On another side reducing sugar (46.277 mg/g) on mean basis was observed highest followed by 46.108 mg/g at 6h which is statistically at par.

Table: 3 Interaction of table Variety and Incubation period

variety	1h	2h	3h	4h	5h	6h	7h	Mean
MTU-1010	11.687	21.121	28.327	29.128	45.016	45.888	46.005	32.453
IR-64	12.041	21.735	28.907	36.942	39.289	46.051	45.999	32.995
IR-36	12.078	21.600	28.700	36.951	45.484	46.851	47.278	34.135
DANTESHWARI	11.721	21.142	28.323	36.619	39.986	45.642	45.824	32.751
Mean	11.882	21.400	28.564	34.910	42.444	46.108	46.277	

	C.D.	SE(m)
Variety	0.334	0.120
Time	0.442	0.159
Interaction	0.885	0.317

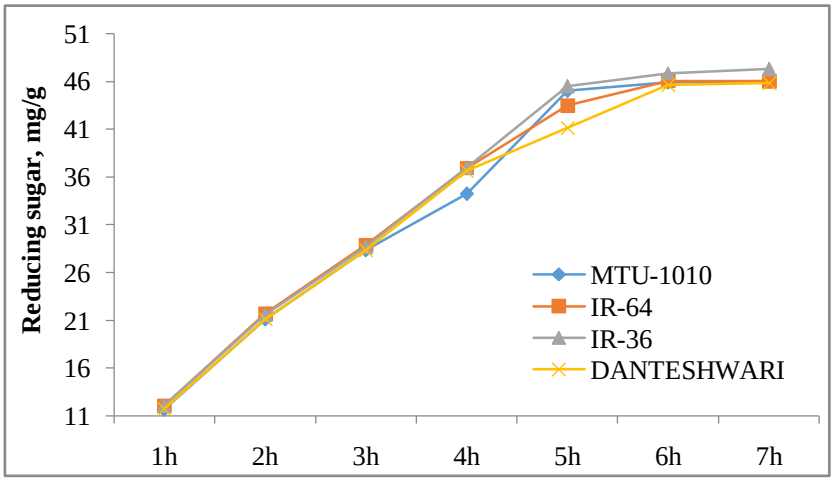


Fig. 7 Interaction of Variety and Incubation period

Effect of Incubation period on releasing of reducing sugar at different enzymatic concentration pre-treatment

Table 4 and Fig. 8 indicate that maximum reducing sugar was released with 2% enzyme concentration treatment ranging from 14.636 to 68.060 mg/g at different incubation period, with the mean 46.456 mg/g. However, 46.365 mg/g was observed with 1% enzyme treatment which is statistically at par. On other hand highest (46.277 mg/g) reducing sugar on mean basis was released after 7h incubation period; however, at 6h, 46.108 mg/g on mean basis was observed which is at par.

Table: 4Interaction table incubation period and concentration enzymatic treatment

Treatment	1h	2h	3h	4h	5h	6h	7h	Mean
0%	5.126	5.177	5.190	5.236	5.251	5.294	5.294	5.224
0.5%	13.235	25.993	34.998	38.088	40.062	43.750	43.897	34.289
1%	14.530	27.108	36.867	48.019	62.348	67.827	67.856	46.365
2%	14.636	27.321	37.203	48.297	62.114	67.561	68.060	46.456
Mean	11.882	21.400	28.564	34.910	42.444	46.108	46.277	

	C.D.	SE(m)
Enzyme treatment	0.334	0.120
Time	0.442	0.159
Interaction	0.885	0.317

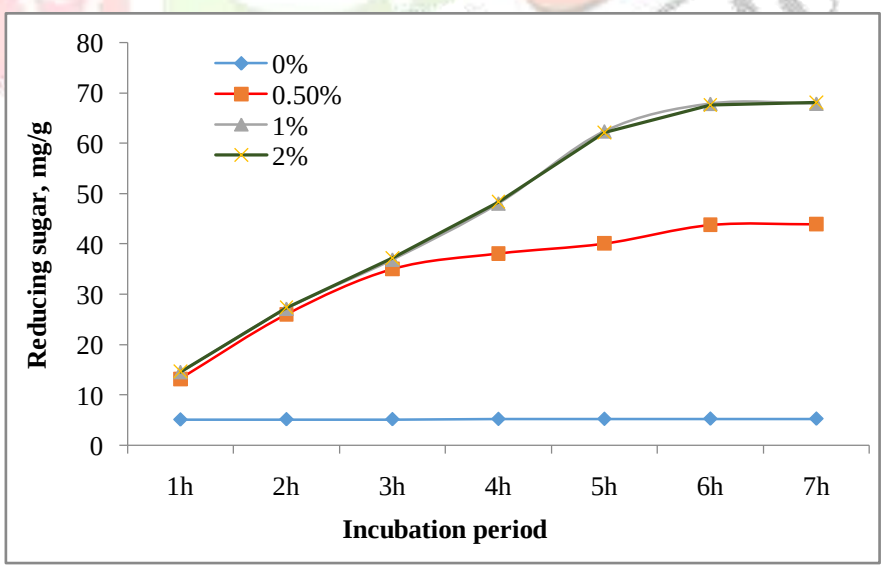


Fig. 8 Interaction of incubation period and treatment of enzymatic concentration

Effect of enzyme concentration on reducing sugar content at different rice varieties

Sugar content was highest from 5.269 to 48.237 mg/g (Table 5 and Fig. 9) with all the enzyme concentration in IR-36, with the mean 34.135 which is significantly higher in comparison to other rice varieties. On other hand highest (46.456 mg/g) reducing sugar content on mean basis was found in 2% enzyme concentration; however, 46.365 mg/g at 6h was statistically at par.

Table: 5Interaction of table variety and enzymatic concentration

Variety	0%	0.5%	1%	2%	Mean
MTU-1010	5.194	34.319	44.995	45.304	32.453
IR-64	5.240	33.842	46.477	46.420	32.995
IR-36	5.269	34.722	48.310	48.237	34.135
DANTESHWARI	5.192	34.273	45.677	45.863	32.751
Mean	5.224	34.289	46.365	46.456	

	C.D.	SE(m)
Variety	0.334	0.120
Enzyme treatment	0.334	0.120
Interaction	0.669	0.240

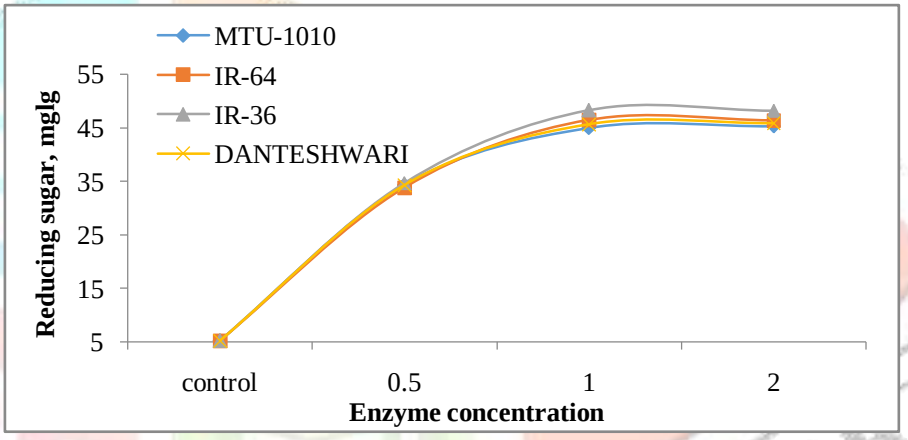


Fig. 9 Interaction variety and enzymatic concentration

The results of the investigation (Table 6 and Fig.10) clearly revealed that reducing sugar content in control (zero per cent concentration) was 5.330 mg/g even at 7h. Maximum sugar was observed at 7h incubation period with 2% enzyme treatment in IR-36 rice variety. However, sugar content 69.920 mg/g and 69.952 mg/g with 1% enzyme treatment at 6h and 7h respectively in the same IR-36 rice variety is statistically at par.

Referring the ANOVA (Table 7) it was observed that the varieties, enzyme concentration, incubation period and their interactions had significant effect on the release of reducing sugar at 5 % confidence level.

Hydrolysis of starch was carried out using enzyme treatment. In the above experiment rice starch was hydrolyzed using various concentration of α-amylase enzyme. The enzymatic hydrolysis of different biomass depends upon different parameters *viz.*, structural property of the substrate, bonding mode of action for enzyme, adsorption and desorption phenomenon (Sattler *et al.*, 1998). Enzyme digests the starch at faster rate than the acid treatment as revealed from the above results. As the concentration of enzyme is increases the amount of free sugar increases up to a limit, where other factor limits the enzyme activity as shown from the result that sugar content was significantly higher at 1% enzyme treatment in comparison to 0.5%. However, the sugar content released by 1% enzyme was statistically at par to the sugar content at 2% enzyme treatment. Starch quality also affects the enzyme activity. Similar work was carried out by Aguirre *et al.* (1978) and they reported that 0.1 per cent of α-amylase gives best results when tested on processing of pre-cooked rice and maize flours at different concentration.

Table: 7 Analysis of variance (ANOVA) table for effect of enzyme treatment, incubation period on reducing sugar in different rice varieties

Source of Variation	DF	Mean squares	F- Cal	C.D.	SE(m)
Variety (A)	3	45.346	37.515	0.334	0.120
Enzyme treatment(B)	3	31,718.721	26,240.839	0.334	0.120
Int. AxB	9	11.466	9.486	0.669	0.240
Time (C)	6	8,328.795	6,890.396	0.442	0.159
Int. AxC	18	45.349	37.517	0.885	0.317
Int. BxC	18	1,238.691	1,024.767	0.885	0.317
Int. (AxBxC)	54	13.098	10.836	1.769	0.635
Error	224	1.209			
Total	335				

Similarly, Brooks and Griffin (1987) observed maximum reducing sugars for both long and short grain rice varieties at 0.01 per cent (w/v) concentration and 70ºC temperature. Complex starch (higher percent of amylopactine ratio with higher degree of branching) is less digested by the enzyme. Starch quality differs from variety to variety. Above results also reveals that there is a significant variation in release of free sugar among the varieties. Hence, from the above results it is inferred that reducing sugar was maximum in rice variety IR-36 followed by other rice variety. It was also cleared from the above results that enzyme treatment @ 2% was better but treatment @ 1% was at par. Similarly, the incubation period 7h gives highest amount of free sugar; however, 6h was at par.

Ethanol Production

Saccharomyces cerevisiae strains are known for ethanol production from various carbohydrates containing raw material. In this experiment raw material used for ethanol production was broken rice after pre-treatment (various percent of α-amylase treatment for 6h). Pre-treated rice from all the varieties was further incubated with three different yeast strain of *Saccharomyces cerevisiae* namely: *viz.* NCIM 3570, NCIM 328 and NCIM 3640 for ethanol production. The ethanol produced after fermentation was analysed using standard method and ethanol content presented on percent basis.

Effect of yeast strain on ethanol production from different varieties

Table 8 and Fig. 11 indicate that maximum ethanol production was found in IR-36 ranging from 4.064 to 4.039% with all three different cultures, with the mean 4.063 which is significantly higher in comparison to other rice varieties, while IR-64 produces least ethanol (3.862%) on the mean basis. On other hand significantly higher ethanol (4.039) percentage on mean basis was produced with yeast strain NCIM 3281.

Table: 8 Interaction table of different culture and rice varieties

Variety	NCIM 3570	NCIM 3281	NCIM 3640	Mean
MTU-1010	4.013	3.998	4.005	4.005
IR-64	2.766	4.038	3.781	3.862
IR-36	4.064	4.085	4.039	4.063
DANTESHWARI	4.014	4.037	4.019	4.023
Mean	3.964	4.039	3.961	
			C.D.	SE(m)
		Variety	0.010	0.004
		Culture	0.009	0.003
		Interaction	0.018	0.006

Table: 6 Mean Table for the effect of Enzyme pre-treatment, Incubation period and Rice varieties on releasing of reducing sugars

	MTU-1010				IR-64				IR-36				DANTESHWARI			
	0%	0.5%	1%	2%	0%	0.5%	1%	2%	0%	0.5%	1%	2%	0%	0.5%	1%	2%
1h	5.109	12.973	14.382	14.382	5.117	13.464	14.775	14.808	5.198	13.956	14.710	14.808	5.080	12.907	14.349	14.546
2h	5.153	25.690	26.673	26.968	5.183	26.313	27.722	27.722	5.235	26.280	27.296	27.591	5.139	25.690	26.739	27.001
3h	5.168	34.834	36.441	36.867	5.161	35.261	37.522	37.686	5.249	35.162	37.063	37.325	5.183	34.736	36.441	36.932
4h	5.190	37.950	36.539	36.932	5.279	38.407	51.944	52.140	5.279	38.440	51.911	52.173	5.198	37.653	51.681	51.944
5h	5.190	41.357	66.726	66.791	5.286	35.392	58.722	57.756	5.294	42.045	67.316	67.283	5.235	41.455	56.627	56.627
6h	5.271	43.684	67.086	67.512	5.327	43.979	67.447	67.450	5.330	43.684	69.920	68.468	5.249	43.651	66.855	66.813
7h	5.279	43.848	67.217	67.676	5.330	44.077	67.211	67.377	5.301	43.848	69.952	70.013	5.264	43.815	67.043	67.174

	C.D.	SE(m)
Variety	0.334	0.120
Enzyme treatment	0.334	0.120
Time	0.443	0.159
Interaction Variety, Treatment and Time	1.769	0.635

(Note: B1, B2, B3 and B4 represent enzyme concentration 0%, 0.5%, 1% and 2% respectively and C1, C2, C3, C4, C5, C6 and C7 represent incubation period 1h, 2h, 3h, 4h, 5h, 6h and 7h respectively)

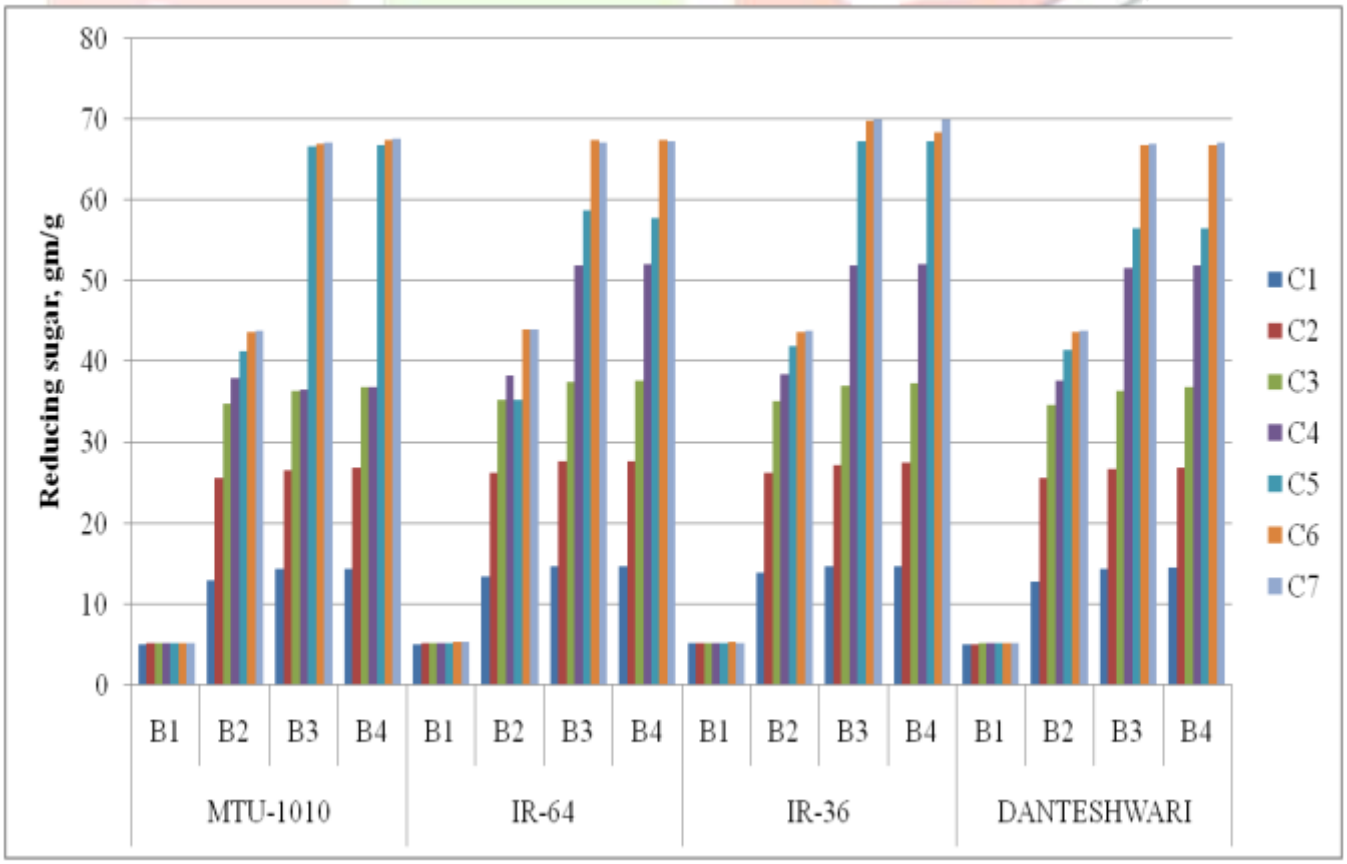


Fig. 10 Effect of Enzyme pre-treatment, Incubation period and Rice varieties on releasing of reducing sugars

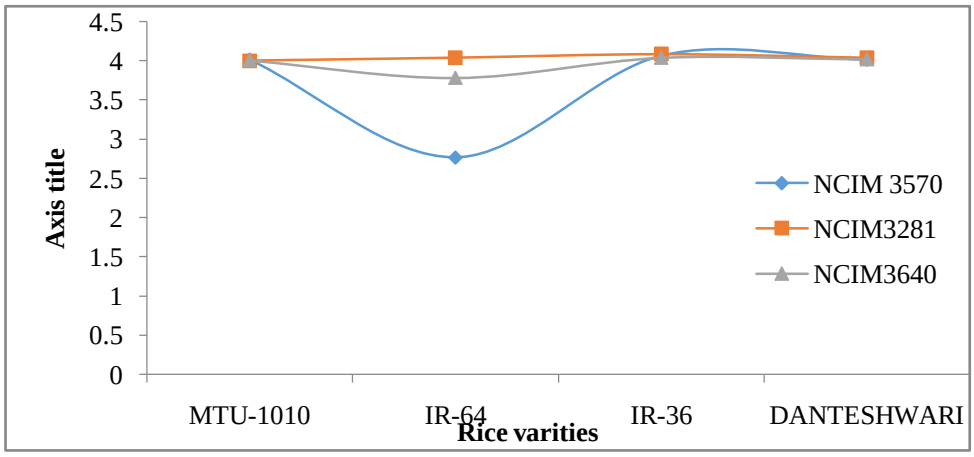


Fig. 11 Interaction of different culture and rice varieties

Effect of enzymatic concentration on ethanol production in different rice varieties

From the Table 9 and Fig. 12 it can be inferred that maximum ethanol production is found in IR-36 ranging from 0.494 to 6.349% with different concentration of enzyme, with the mean 4.063% which is significantly higher in comparison to other rice varieties. On other side highest ethanol (6.307) percentage on mean basis was observed with pre-treatment of 1 % enzyme concentration for 6h.

Table: 9 Interaction table of enzyme concentration and rice variety

Variety	C1	C2	C3	C4	Mean
MTU-1010	0.495	2.982	6.257	6.286	4.005
IR-64	0.493	2.953	6.338	5.663	3.862
IR-36	0.494	3.067	6.340	6.349	4.063
DANTESHWARI	0.492	2.982	6.294	6.325	4.023
Mean	0.494	2.996	6.307	6.156	

	C.D.	SE(m)
Variety	0.010	0.004
Enzyme treatment	0.010	0.003
Interaction	0.021	0.007

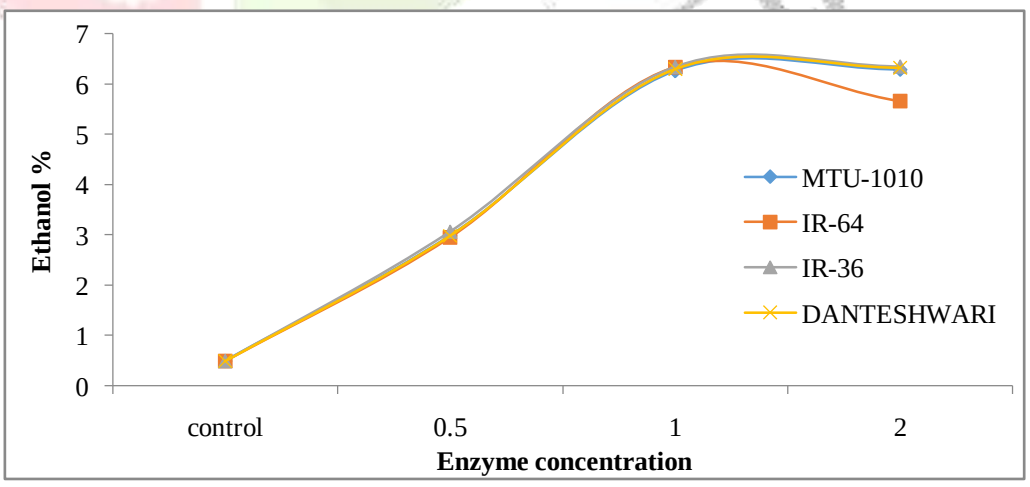


Fig. 12 Interaction enzyme concentration and rice variety

Effect of enzymatic concentration on ethanol production in different culture

Results from the Table 10 and Fig. 13 indicate that maximum ethanol is produced with the culture NCIM 3281 ranging from 0.494 to 6.337% in substrate treated with different concentration of enzyme, with the mean 4.039% which is significantly higher in comparison to other cultures. On other side highest ethanol (6.307%) on mean basis was produced in the substrate treated with 1% enzyme concentration up to 6h.

Table: 10 Interaction table of culture and treatment

Culture	0%	0.%	1%	2%	Mean B
NCIM 3570	0.495	2.965	6.313	6.083	3.964
NCIM 3281	0.494	3.018	6.310	6.337	4.039
NCIM 3640	0.492	3.005	6.298	6.048	3.961
Mean C	0.494	2.996	6.307	6.156	

	C.D.	SE(m)
Culture	0.009	0.003
Enzyme treatment	0.010	0.004
Interaction	0.018	0.006

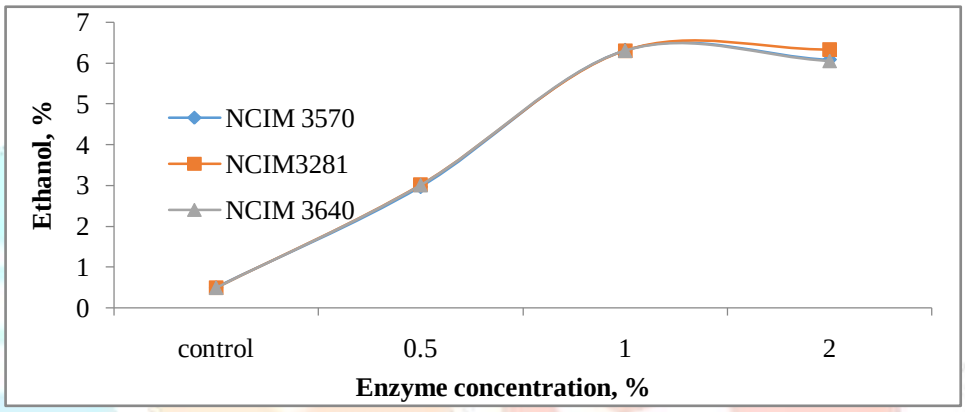


Fig. 13 Interaction of culture and treatment

Effect of different culture on ethanol production from enzymatic pre-treated different rice varieties

Rice varieties differ with respect to production of ethanol content (Table 11 and Fig. 14). The amount of ethanol was found to be significantly higher from IR-36 variety (6.386%). While, the maximum production of ethanol from variety IR-64 and Danteshwari was 6.336 % and 6.335%. From the results it is also revealed that the yeast strain NCIM 3281 produce highest ethanol in all the varieties.

Ethanol is produced by the yeast through fermentation process. Yeast strain differs in their capacity to produce ethanol and ethanol production from the yeast strain also affected by the other factors. In the above experiment three yeast strains were incubated with substrate from four different rice varieties treated at four different enzyme concentrations. From the results of the above experiment it is revealed that rice variety IR-36 treated with 1% α-amylase enzyme produce significantly higher ethanol (6.386%) with NCIM 3281 strain, while IR-64 produce least amount of ethanol.

Referring the ANOVA (Table 12) it was observed that the varieties, enzyme treatment and yeast strain along with their interactions significantly affect the ethanol production at 5% confidence level.

Table: 12 Analysis of variance (ANOVA) table for ethanol production with different cultures and enzymatic treatments in different rice varieties

Source of Variation	DF	Mean squares	F- Cal	C.D.	SE (m)
variety (A)	3	0.278	566.991	0.010	0.004
Culture (B)	2	0.096	195.964	0.009	0.003
Int. AxB	6	0.064	131.199	0.018	0.006
Enzyme% (C)	3	279.276	570507.832	0.010	0.004
Int. AxC	9	0.246	502.232	0.021	0.007
Int. BxC	6	0.071	144.124	0.018	0.006
Int. (AxBxC)	18	0.061	124.155	0.036	0.013
Error	96	0.000			
Total	143				

Table: 11 Mean Table for the effect of Enzyme pre-treatment, different cultures and Rice varieties on ethanol production

	MTU-1010			IR-64			IR-36			DANTESHWARI		
	B1	B2	B3	B1	B2	B3	B1	B2	B3	B1	B2	B3
C1	0.496	0.495	0.493	0.496	0.493	0.490	0.495	0.495	0.493	0.492	0.492	0.492
C2	2.965	2.958	3.023	2.914	2.987	2.958	3.052	3.110	3.038	2.929	3.016	3.001
C3	6.277	6.248	6.248	6.335	6.336	6.342	6.343	6.349	6.328	6.298	6.306	6.277
C4	6.313	6.291	6.255	5.320	6.335	5.333	6.344	6.386	6.298	6.335	6.335	6.306

	C.D.	SE(m)
Variety	0.010	0.004
Culture (B)	0.009	0.003
Enzyme treatment (C)	0.010	0.004
Interaction AxBxC	0.036	0.013

(Note: C1, C2, C3 and C4 represent different enzyme concentration 0%, 0.5%, 1% for pre-treatment respectively and B1, B2 and B3 represent cultures NCIM 3570, NCIM 3281 and NCIM 3640 respectively)

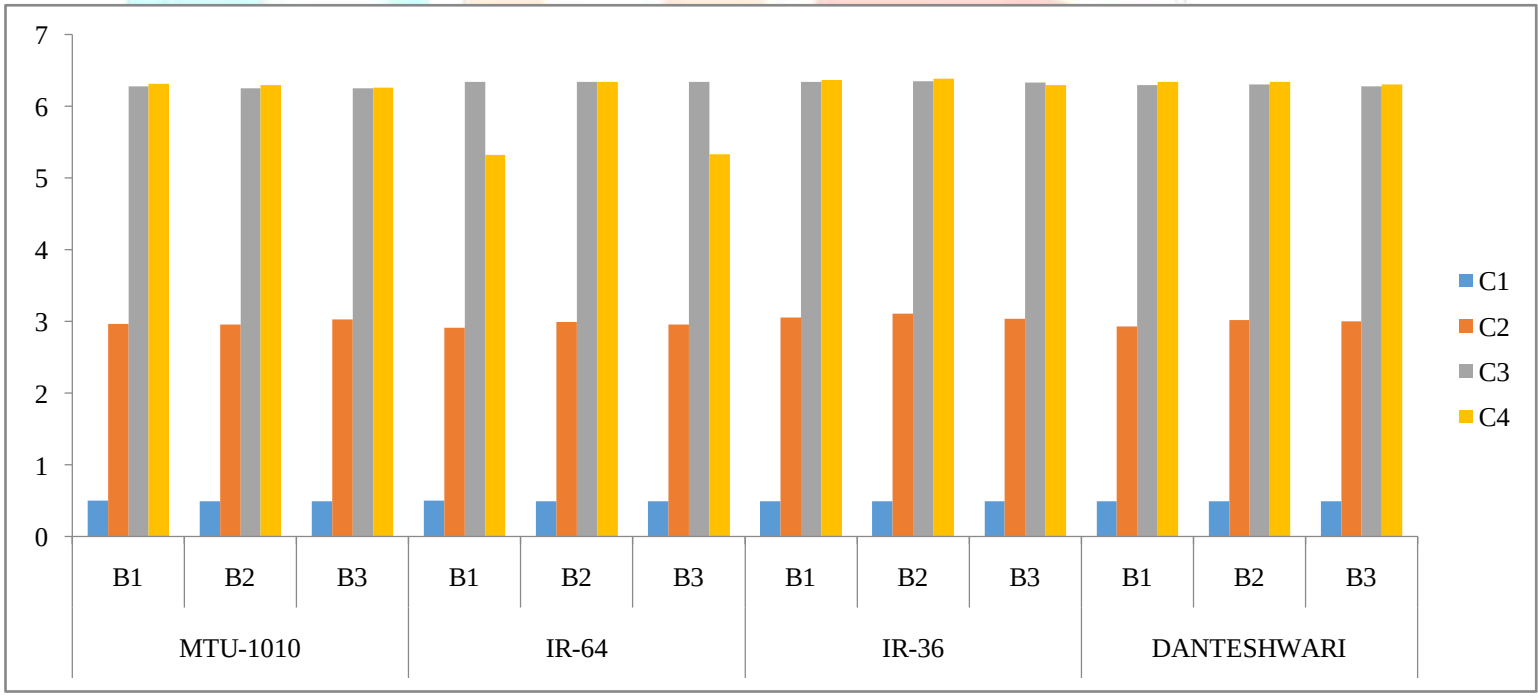


Fig. 14 Effect of Enzyme pre-treatment, different cultures and Rice varieties on ethanol production

Ethanol production at optimized conditions

Ethanol was produced by following all the optimized conditions from IR-36 with *S.cerevisiae* NCIM 3281 and it was recorded 6.858%.

Table: 13 Ethanol production at optimized condition

Rice	Substrate concentration	Culture	Temperature	Agitation	Ethanol %
IR-36	1:1	NCIM 3281	30±1 ºC	100 rpm	6.858

SUMMARY AND CONCLUSION

Summer season rice is grown in large area of Chhattisgarh and it is known that this type of rice has higher broken percentage on milling in comparison to *kharif* grown rice. Losses are also incurred during storage due to various other factors. A large quantity of different grains is spoiled every year in India because of un-favourable climatic conditions, inadequate transport and storage facilities. These broken, spoiled grains from rice varieties, which are less preferred or not preferred commercially because of boldness in grains, not of fine quality and stickiness can be used for preparation of fermented products, which can generate employment opportunities and income.

In the present investigation, four rice varieties were selected out of six on the basis of higher broken percentage for the experiment. Starch and protein content were analyzed for selected four varieties. Broken rice from these varieties were pre-treated with α-amylase enzyme for the release of reducing sugar as the sugar is used by the microbial culture for its growth and production of ethanol. For ethanol production three yeast cultures out of twenty-three (*Saccharomyces cerevisiae* NCIM 3281, *Saccharomyces cerevisiae* NCIM 3570 and *Saccharomyces cerevisiae* NCIM 3640) were selected and procured from national repositories on the basis of ethanol production. These strains were used for fermentation with the substrate from all four varieties. After finalization of yeast strain along with the variety the conditions were optimized for maximum production of ethanol.

From the results it was observed that there was no significant difference in starch and protein content between the varieties. The highest starch content (84.393%) was recorded in IR-36 variety, followed by IR-64 variety (83.067%), while protein content was highest (7.997%) in IR-64 variety, followed by IR-36 variety and Danteshwari variety recorded lowest protein content (7.200%).

There was significant difference between the varieties and significantly higher reducing sugars was recorded in IR-36 variety (69.920 mg/g). Variety IR-64 and MTU-1010 recorded 67.447 and 67.086 mg/g respectively. The commercial α-amylase was significantly effective at 1% concentration for 7 hours of incubation time in releasing maximum reducing sugars in all the rice varieties except IR-64. However, 6h treatment was statistically at par.

From the pre-treatments experiment used for hydrolysis, commercial α-amylase pre-treatment up to 6h was selected for further hydrolysis and ethanol preparation from different rice varieties.

Yeast strain *S. cerevisiae* NCIM3570, NCIM 3281, and NCIM 3640 were used at 5 percent inoculum level. In all the experiments yeast strain NCIM 3281 produced highest ethanol (6.349%) and in all four rice varieties highest ethanol (6.349%) was produced with IR-36, while least amount of ethanol was produced from IR-64. IR-64 variety produced significantly varied amount of ethanol with all three cultures.

CONCLUSION

1. The commercial α-amylase pre-treatment giving maximum reducing sugars was suitable for ethanol preparation using the *Saccharomyces cerevisiae.*
2. *Saccharomyces cerevisiae* NCIM 3281 culture produced highest percent (6.349) of ethanol with IR-36 rice variety followed by NCIM 3570.
3. Optimum temperature for ethanol (6.858%) production is 30 ± 1^0C is with *Saccharomyces cerevisiae* NCIM 3281 using rice from IR-36 at agitation speed 100 rpm and pre-treated substrate diluted at 1:1 ratio.

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Lebaka Veeranjaneya Reddy1*, Obulam Vijaya Sarathi Reddy2 and Young-Jung Wee3* 1. Department of Microbiology, Yogi Vemana University, Kadapa (A.P.) 516003, India. 2. Department of Biochemistry, Sri Venkateswara University, Tirupati 517502, India. 3. Department of Food Science and Technology, Yeungnam University, Gyeongsan, Gyeongbuk 712-749, Korea. Accepted 4 April, 2011

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